ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1979, p. 638-371
0066-4804/79/03-0368/04$02.00/0
Vol. 15, No. 3

Avermectins, New Family of Potent Anthelmintic Agents: Isolation and Chromatographic Properties

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Received for publication 15 November 1978

The avermectins, a family of new anthelmintic agents, were isolated from the mycelia of *Streptomyces avermitilis*. Four closely related major components and four homologous minor components were separated from the complex. Solvent extraction, solvent partition, and adsorption methods were used to isolate and purify the complex; novel partition chromatography systems using Sephadex LH-20 were used to separate the components. A reverse-phase high-pressure liquid chromatography assay for the quantitative determination of all components was used extensively to monitor the purification methods.

The avermectin complex, a family of new anthelmintic agents, is produced by fermentation of *Streptomyces avermitilis* MA-4680 (NRRL 8165), as described in a separate publication (1).

The complex contains four closely related major components, A_{1a}, A_{2a}, B_{1a}, and B_{2a}, in varying proportions and four minor components, A_{1b}, A_{2b}, B_{1b}, and B_{2b}, each of which is a lower homolog of the corresponding major component. The structures and physicochemical properties of all components are the subject of a separate publication (G. Albers-Schönberg, B. H. Arison, J. C. Chabala, A. W. Douglas, P. Eskola, M. H. Fisher, J. M. Hirschfield, K. Hoogsteen, A. Lusi, H. Mrozik, J. L. Smith, J. P. Springer, and R. L. Tolman, J. Am. Chem. Soc., in press).

In this report, the isolation of the complex from broth, the separation of all of its components, and the chromatographic properties of each will be described. For the sake of simplicity, mixtures of homologs will be designated by omission of the second letter; for example, B_1 will indicate a mixture of B_{1a} and B_{1b}.

(This paper was presented in part at the 18th Interscience Conference on Antimicrobial Agents and Chemotherapy, 1-4 October 1978, Atlanta, Ga.)

MATERIALS AND METHODS

Bioassays and TLC assays. Initial purification studies were guided first by animal assays and later by thin-layer chromatography (TLC) assays as described in the companion publication (1).

HPLC assays. The system developed for the quantitative analyses of avermectins employed a Waters Associates 10-μm Bondapak C_{18} reverse-phase column (0.4 by 30 cm) maintained at 40°C, a solvent system of methanol-water (85:15, vol/vol), and a flow rate of 1.2 ml/min. A Waters Associates model 6000A pump and model U6K loop sample injector were used. The column effluent was monitored by a Schoeffel Instrument Corp. model SF770 ultraviolet (UV) monitor set at 246 nm and 0.2 absorbance units full scale. The monitor output was coupled to a Honeywell model 195 strip chart recorder for visual observation and a Spectra-Physics System I digital computing integrator for quantitative results. Samples of 5 μl, containing 1 μg to 2 mg of each component per ml, were injected. The elution volumes (in milliliters) for the eight components in their order of elution were as follows: B_{2b}, 6.14; B_{2a}, 6.74; A_{2b}, 7.22; A_{2a}, 8.00; B_{1b}, 8.32; B_{1a}, 9.70; A_{1b}, 11.06; and A_{1a}, 11.78. The UV monitor was set at 246 nm to improve the resolution of a major impurity which exhibited a UV maximum at a lower wavelength. Although the UV maximum for avermectins occurred at 245 nm, sensitivity was only slightly reduced at 246 nm, whereas the sensitivity for the impurity was reduced substantially. The UV spectrum of B_{2a} is given in Fig. 1 and is characteristic of all components. Recordings of high-pressure liquid chromatography (HPLC) assays of standard and unknown solutions are given in Fig. 2.

RESULTS

Isolation of avermectin complex. Approximately 8,000 liters of fermentation broth was filtered, and the cake was washed with water. The wet cake was then slurried in 3,000 liters of acetone, filtered, and washed with 400 liters of 80% acetone. The cake was discarded, and the combined extract and wash were concentrated to 800 liters. The concentrate was adjusted to pH 5 with dilute HCl and then extracted twice with 800-liter portions of methylene chloride.

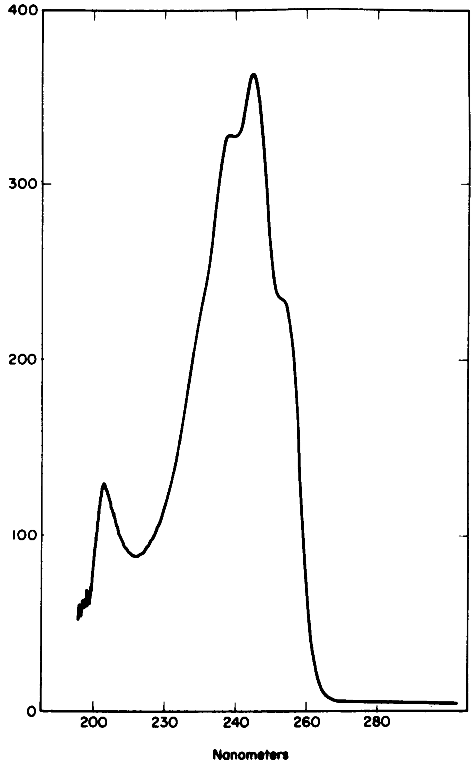

FIG. 1. *UV absorption spectrum of avermectin B_{2a} in methanol.*

The extracts were combined and dried by filtering through a pad of dry Super-Cel, followed with a wash of 40 liters of methylene chloride. The filtrate was concentrated under reduced pressure to a small volume, and methanol was added while the concentration continued until the methylene chloride was removed and a final volume of 38 liters remained.

The methanol concentrate was added to 95 liters of ethylene glycol, and the mixture was then extracted with 130 liters of heptane. The heptane layer was separated and extracted with a mixture of 18 liters of ethylene glycol and 6 liters of methanol. The two ethylene glycol-methanol extracts were combined, diluted with 150 liters of ethyl ether, and washed with 150 liters of water containing 8% NaCl. After separation, the ether layer was washed further with three 75-liter portions of water containing 2% NaCl. The water washes remove ethylene glycol from the extracts, and salt helps to prevent emulsion formation. The washed ether extract was concentrated under reduced pressure to a small volume, and methylene chloride was added while the concentration continued until the ether was removed and a final volume of 34 liters remained.

The methylene chloride concentrate was passed through a column containing 34 kg of acid-washed alumina as a bottom layer and 34 kg of Pittsburgh CAL granular carbon as a top layer, both slurried in methylene chloride. The column was washed with 34 liters of methylene chloride followed by 680 liters of methylene chloride containing 3% isopropyl alcohol by volume. The column was eluted at 400 ml/min, and the first 68 liters of effluent was discarded. The remainder of the effluent was concentrated under reduced pressure until no further distillate appeared. The oily residue weighed 2.3 kg and contained about 5% A_1, 16% A_2, 20% B_1, and 15% B_2 components.

Separation of A components from B components. A 100-liter partition column was prepared by swelling 30 kg of Sephadex LH-20 in methanol and pouring the slurry into a column. The resin was then equilibrated with the partition solvent mixture hexane–methylene chlo-

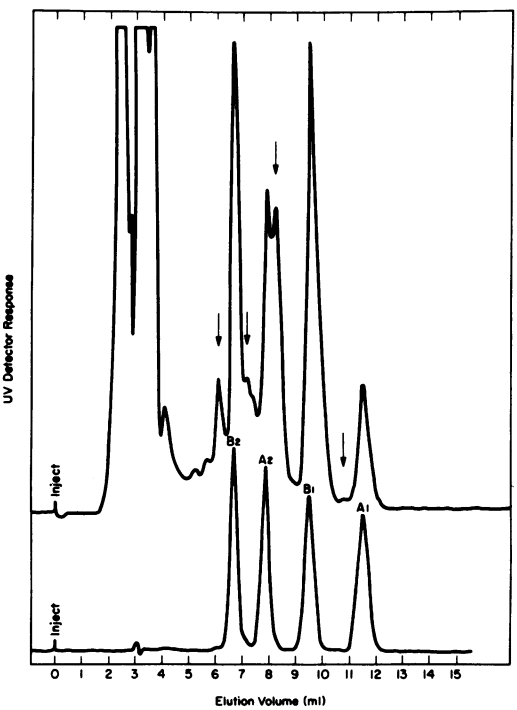

FIG. 2. *HPLC curves. The lower curve is the UV trace resulting from an injection of 5 µl of a solution containing 1.00 mg of each of the major components per ml. The upper curve is from an injection of 5 µl of acetone extract of mycelia at a dilution of 1:1.2 of whole broth. Peaks corresponding to the minor components are indicated by arrows, each minor component eluting slightly before its counterpart in the series of major components. Column conditions are given in the text.*

ride-methanol (10:10:1, by volume). The product from the previous step was dissolved in the partition solvent mixture, and samples equivalent to 500 g of crude were applied to the column and eluted with the same solvent mixture at 400 ml/min. A forerun of 40 liters was collected, and then 20 fractions of 2 liters each were collected and analyzed by TLC and HPLC. The elution peak of the A components occurs at about 0.5 column volume, and that of the B components occurs at about 0.7 column volume. Appropriate fractions were pooled based on analyses, and the solvent was removed by evaporation at a reduced pressure. The residue containing A components weighed 444 g and contained about 23% A_1 and 64% A_2, whereas the residue containing B components weighed 723 g and contained about 57% B_1 and 43% B_2. An elution profile determined by HPLC assays is given in Fig. 3.

Separation of A_1 from A_2. Another 100-liter partition column was prepared by swelling 30 kg of Sephadex LH-20 in methanol and pouring the slurry into a column. The resin was then equilibrated with the partition solvent mixture hexane-toluene-methanol (6:1:1). A 400-g portion of the residue containing A components from the previous step was dissolved in the above solvents in the proportions 3:1:1, applied to the column, and eluted with a 6:1:1 proportion at 400 ml/min. Fractions were collected and analyzed as before. The eluate peak of A_1 occurs at about 0.6 column volume, and that of A_2 occurs at about 1.6 column volumes. Products were recovered as before to yield 40 g of A_1, 96% pure, and 160 g of A_2, 97% pure. Intermediate fractions containing mixed components were recovered for repeating the separation.

Separation of B_1 from B_2 by crystallization. Component B_1 crystallizes readily from many organic solvents; however, the yield of B_1 and the degree of separation from B_2 vary with the solvent. Crystallization from ethylene glycol afforded both high yield and good separation. A 619-g portion of the mixed B components was dissolved in 2 liters of methylene chloride to insure complete dissolution of the product. The methylene chloride was distilled off while 13.6 liters of methanol was added continuously to produce a hot methanol solution of B components. The methanol was then distilled off under reduced pressure while 13.6 liters of ethylene glycol was added continuously until most of the methanol was removed. The resulting slurry was cooled and filtered to separate crystals rich in B_1 from mother liquors rich in B_2. The crystals were recovered and recrystallized twice in a similar fashion to yield 217 g of B_1, 97% pure. The ethylene glycol mother liquors were extracted with toluene to recover B_2 from the nonvolatile solvent.

Separation of B_1 from B_2 by chromatography. A slightly different partition system was developed for the separation of B_1 from B_2. The method is identical to that used for the separation of A_1 from A_2 except that the proportions of hexane-toluene-methanol were 3:1:1. In this system the eluate peak of B_1 occurs at about 0.9 column volume, and that of B_2 occurs at about 1.05 column volumes. The B_2 fraction recovered from crystallization mother liquors, 336 g, was dissolved in the solvent mixture above, applied to a 100-liter column, and developed with the same solvent mixture. Fractions were selected based on HPLC and TLC analyses, pooled, concentrated to an oil under reduced pressure, and crystallized from benzene to yield 106 g of B_2, 99% pure. This partition system was also used to separate B_1-B_2 mixtures before crystallization. An elution profile is given in Fig. 4.

Separation of B_{1a} from B_{1b} by chromatography. The HPLC analyses of fractions in the B_1 peaks eluted from the Sephadex LH-20 columns with solvent systems containing hexane-toluene-methanol indicated a partial separation of B_{1a} and B_{1b}. An 8.5-liter column of Sephadex LH-20 was prepared in these solvents in the proportion 3:1:1, and 7.4 g of B_1 contain-

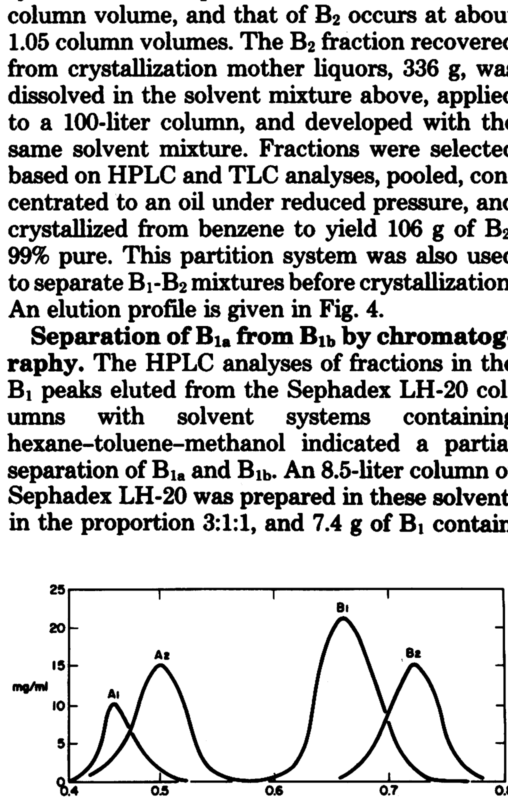

FIG. 3. *Sephadex LH-20 chromatography for the separation of A components from B components. The solvent system is hexane-methylene chloride-methanol (10:10:1).*

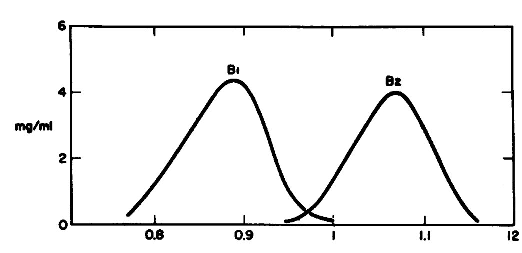

FIG. 4. *Sephadex LH-20 chromatography for the separation of B_1 from B_2. The solvent system is hexane-toluene-methanol (3:1:1).*

ing 92% B_{1a} and 8% B_{1b} was charged and developed with the same solvent. Appropriate fractions were pooled based on HPLC analyses, concentrated, and rechromatographed. Appropriate fractions from the second run were pooled, concentrated, and crystallized from ethanol to yield B_{1a}, 99.5% pure. The elution peaks of B_{1a} and B_{1b} for this column were found to occur at 0.83 and 0.86 column volume respectively. An elution profile is given in Fig. 5.

The yield of pure B_{1a} from this step depends on the composition of the initial sample, the efficiency of the column, and the selection of fractions. Separation of major and minor components of the other three mixtures was achieved by this system or the variation containing the solvent in the proportion 6:1:1.

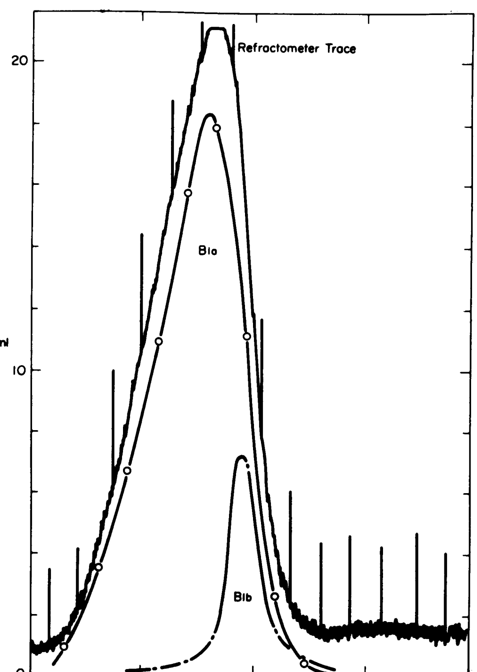

FIG. 5. *Sephadex LH-20 chromatography for the separation of B_{1a} from B_{1b}. The solvent system is hexane-toluene-methanol (3:1:1).*

DISCUSSION

This report describes a practical scheme for the isolation of all of the components of the avermectin complex. The initial separation methods were guided by mouse assays until a correlation was established between the UV absorption spectrum and anthelmintic activity. After a potent concentrate was prepared, TLC revealed the presence of four active components. A TLC assay system was used for an interim period (1), and small amounts of separate components were isolated by preparative TLC. The HPLC assay was then developed to resolve all eight components and to quantitate each, and most of the separations described above were monitored by this method.

Preparative HPLC was used to separate additional quantities of mixed components; however, the poor solubility of avermectins in the developing solvent, 85% methanol, limits this method. The solvent mixtures developed for Sephadex LH-20 chromatography provide high capacity as well as good resolution of components. The HPLC assay was used as the major criterion of quality, and all of the major components were obtained at least 99% pure for biological evaluation (2).

ACKNOWLEDGMENTS

We express our appreciation to the many members of Merck Sharp & Dohme Research Laboratories who contributed to this work.

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